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**Rapid Measurement of Protease Activity Prevalent in  
Bacteria from Wounds: A Diagnostic for Total Bioburden**

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Key Words:	proteolytic enzyme, protease, infection, bioburden, diagnostic



**Rapid Measurement of Protease Activity Prevalent in Bacteria from Wounds: A Diagnostic for Total Bioburden.**

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## ABSTRACT

The point-of-care diagnosis of localized wound infection is problematic because current strategies to measure bioburden rely on culture-based methods which can take at least 48 hours to obtain results. Bacterial protease activity appears to be a promising marker for the rapid and simple detection of bacteria in wound samples. Using a peptide substrate that is clipped by a broad spectrum of the bacterial species prevalent in wounds, it was shown that over 80% of wound isolates of *S. aureus*, *E. faecalis*, *S. pyogenes*, and *P. aeruginosa* produced proteases *in vitro*. Also, the major protease produced by each of these bacterial species was identified by N-terminal protein sequencing and its cleavage site within the peptide was mapped. The peptide substrate was then incorporated into a novel microbead assay format that enabled the rapid detection of proteolytic activity with a limit of detection that approached  $10^5$  CFU/ml for each of the 23 wound isolates measured. Clinical studies demonstrate that protease activity is present in wounds and can be measured at a level that is reflective of the appropriate bacterial bioburden ( $10^5$  -  $10^6$  CFU/ml) to assist in the diagnosis of infection. Our initial clinical findings also indicate that sensitivity and specificity of detection approaches to 90% and 70% respectively.

INTRODUCTION

Since a wound has compromised innate defenses of the skin and is open to the air, nearly all contain bacteria. However, only in certain instances are the bacteria able to overcome the immune response to slow or stall healing and cause infection. In current practice, the bacterial concentration within the wound bed is considered the most reliable marker of this event (1). The concentration of bacteria, also called bioburden, is typically assessed by quantitative microbiological analysis of a sample collected from the wound. This involves culturing a sample on rich selective media to isolate each prevalent bacterium then enumerating each species (2, 3). The clinical consensus is that a bioburden exceeding  $10^5$  colony-forming units per gram of wound bed tissue (CFU/g) is detrimental to wound healing and thus constitutes an infection (1, 4). This is based on seminal studies demonstrating that healing in burns and pressure ulcers, and the successful outcome of skin graft and flap procedures, depends on maintaining a bacterial level below  $10^5$  per gram of tissue of most bacterial species (5-12).

A significant drawback of assessing bioburden using current laboratory microbiology methodology is the time it takes to obtain results after submission of a sample (1). The culture-based methods, on which bacterial enumeration, identification, and antibiotic sensitivities depend, commonly take 48 hours or more to yield results. The identification of a molecular marker that correlates to bacterial bioburden could enable more convenient and rapid assessment of a wound and provide information at the point-of-care. Currently, no such broad-spectrum molecular marker for bioburden exists.

Virulence factors that contribute to the ability of bacteria to cause infection are obvious candidates for the broad-spectrum detection of bacteria within wounds. The measurement of enzymatic activity from virulence factors has proven to be a reliable method for the detection of bacteria in several infectious applications (13). For instance, determination of bacterial sialidase activity in vaginal samples is said to aid in the diagnosis of bacterial vaginosis. Sialidase is produced by the major causative agents of vaginosis, *Gardnerella vaginalis*,

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3 *Bacteroides spp.*, *Prevotella spp.* and various *Mobilincus spp.*, and the activity of  
4 this enzyme in samples has been shown to correlate with infection as determined  
5 by traditional methods (14). Another example is the detection in gingival  
6 crevicular fluid of proteases produced by periodontal pathogens like  
7 *Porphyromonas gingivalis* and *Tanarella forsythus* (15). This assay measures  
8 bacterial proteolytic activity using a simple colorimetric peptide substrate and  
9 provides information that assists in the diagnosis of periodontal disease.

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16 Proteases are produced by most of the bacterial species present in  
17 wounds including *S. aureus*, *E. faecalis*, *P. aeruginosa*, and *Streptococcus spp.*,  
18 and thus are a candidate marker for the wound bioburden. Secreted proteases  
19 appear to be important virulence factors for several bacterial species and have  
20 been shown to be produced by over 80% of *S. aureus*, *E. faecalis*, *P.*  
21 *aeruginosa*, and *S. pyogenes* strains isolated from patients with a variety of  
22 infection types (16-20). Also, secreted proteases from these bacteria have been  
23 demonstrated to significantly contribute to virulence in animal models of infection  
24 (21-25). A number of protease functions are thought to assist bacterial  
25 pathogens in causing infection and would be important for bacterial persistence  
26 and propagation within the wound environment. Proteases can release free  
27 amino acids from host proteins to satisfy bacterial nutritional requirements,  
28 process and activate bacterial surface adhesins or extracellular toxins, degrade  
29 host extracellular matrix proteins, inactivate immune proteins such as cytokines  
30 and immunoglobulins, and remove host cell-surface receptors (26). Another  
31 potentially important role is to target and degrade antimicrobial peptides (27).

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44 This study is focused on demonstrating that bacterial protease activity is a  
45 valid candidate marker for the detection of wound bacteria both *in vitro* and *in*  
46 *vivo*. To demonstrate this, we developed a novel microbead-based assay  
47 capable of the rapid measurement of low levels of bacterial protease activity.  
48 The results indicate that protease production by bacteria isolated from wounds is  
49 widespread. Also, the assay is shown to reliably detect proteases produced by  
50 roughly  $10^5$  CFU/ml bacteria, an appropriate level to indicate infection. Our initial  
51 clinical findings indicate that using the level of bacterial protease activity as a  
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biomarker allows for detection of excessive (above the threshold of about  $10^5$  CFU/ml) bioburden with a sensitivity of 90%, and specificity of 70%.

**MATERIALS AND METHODS**

Bacterial strains and growth conditions

Bacterial wound isolates were obtained from clinical microbiology laboratories at the University of Wisconsin Hospital and Clinics (Madison, WI), the Parkland Health and Hospital Systems (Dallas, TX), the University of Colorado Hospital (Denver, CO), and the UCLA Medical Center (Los Angeles, CA). The isolates were obtained using a variety of sampling techniques from wounds of mixed etiology. Strains were initially plated on TSA plus 5% sheep blood. For the measurement of protease activity, *P. aeruginosa* and *P. mirabilis* were grown in nutrient broth, *S. aureus* in V8 medium (28), *E. faecalis* in brain-heart infusion, and *S. pyogenes* in chopped meat medium plus glucose. Cultures were grown at 37°C with shaking at 280 rpm until late logarithmic/early stationary phase. Cell-free growth medium (CFGF) was prepared by centrifugation of the cultures at 13,000xg for 5 minutes and filtration of the supernatant through a 0.2 µm filter. All clinical strains sent from the hospital microbiology labs were identified by strain type only and this study was exempt from requiring IRB approval.

Proteolysis assays

Unlabeled and biotin-polyhistidine-labeled CPI2 and CPI2S (CPI2: GAMFLEAIPMSIPC or CPI2S: GMAFLEAIPC) peptides were obtained from New England Peptide (Gardner, MA). Protease standards endoproteinase Glu-C (V8) and Pseudomonas elastase (Las B) were obtained from EMD Biosciences, La Jolla, CA. For assays analyzing host protease activity, human neutrophil elastase (HNE) was obtained from Athens Research and Technology, Athens, GA. 0.5 µg/ml MMP-2 or MMP-9 (R&D Systems, Minneapolis, MN) were activated overnight with 1 mM p-aminophenylmercuric acetate (APMA) in DMSO.

### Peptide and microbead conjugation

Unlabeled peptide substrates were conjugated to horseradish peroxidase (HRP, Roche) by first reacting HRP with sulfosuccinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (s-SMCC, Pierce) in PBS pH 7.2 for 1 hour. The resulting HRP-maleimide was purified on a P6 gel filtration column (Bio-Rad, Hercules, CA) equilibrated in PBS with 5 mM EDTA. HRP-maleimide was then coupled to a C-terminal cysteine of the peptide (CPI2 or CPI2S) by incubation for 2 hours in PBS with 5 mM EDTA, and the resulting HRP-CPI2/CPI2S was purified on a P6 column. To prepare the HRP-CPI2 or HRP-CPI2S labeled beads, a 500 µl aliquot of Trisacryl beads (CM-Trisacryl M beads, Pall) was rinsed extensively in PBS then mixed with 40 mg of EDC (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride) dissolved in 500 µl 50 mM MES pH 5.2 and 2 mg of HRP-peptide in PBS buffer (roughly 500 µl). This mixture was agitated for 3 hours at room temperature after which the bead conjugate was rinsed three times with PBS. As an alternative approach to using HRP as the reporter tag on the peptide, peptides were synthesized with a biotin and polyhistidine dual affinity tag. Synthesis of dual affinity tag-labeled CPI2s (GAMFLEAIPK(Ahx-biotin)HHHHHH) was performed by New England Peptide (Gardner, MA) utilizing Fmoc solid phase chemistry. The dual affinity tag consists of a long chain Ahx (N-e-Fmoc-e-aminocaproic acid) biotin molecule that was linked to a side chain of a lysine added to the peptide and six histidine residues (a 6xHis tag) added to the peptide C-terminus. This configuration provides sufficient spacing between the two tags to allow binding partner interactions with each to take place. The peptide was purified by reverse phase-HPLC and quality control including mass spectroscopy, HPLC, and amino acid analysis. Trisacryl CM beads (Pall Corporation, East Hills, NY) were prepared for conjugation by rinsing with Dulbecco's phosphate buffered saline (DPBS) plus 3 ppm Proclin 300 (Sigma, St. Louis, MO) three times and then with DPBS without Proclin 300 five times. After each wash, beads were collected by centrifugation. 500 mg of Trisacryl beads were then mixed with 30 mg fresh EDC (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride) in 450 µl of DPBS and 1 mg of

dual affinity tag-labeled CPI2S in 50  $\mu$ l of DMSO. The entire conjugation mixture was rotated for 4 hours at room temperature. The reaction mixture was removed by centrifugation and the beads were blocked with 0.1M ethanolamine for overnight at room temperature with rotation. The conjugated beads were rinsed two times with DPBS with 0.1% polyethylene glycol 5000 (PEG 5000), one time with 0.1% Tween 20 and 0.1% PEG 5000 in DPBS for 15 minutes at room temperature, 6 more times with DPBS with 0.1% PEG 5000, another time with 0.1% Tween 20 and 0.1% PEG 5000 in DPBS for 15 minutes at room temperature, then finally rinsed 6 times with DPBS with 0.1% PEG 5000. Storage was in PBS with 3 ppm Proclin at 4°C. Bead aliquots were rinsed in 100% ethanol (2x) and placed into sterile tubes. The ethanol was removed after collection of the beads by centrifugation, and the aliquots were dried in a convection oven at 40°C and stored at 4°C.

HRP-CPI2-Trisacryl or HRP-CPI2S-Trisacryl proteolysis assay

To test correlation of the assay to protease concentration and limit of detection, the bacterial concentration (CFU/ml) in overnight cultures was estimated by  $A_{600}$ . The bacterial cells were removed, and the concentration was adjusted as indicated by dilution into fresh growth medium. Initial estimates of bacterial concentration were confirmed by dilution plating. CFGM samples were tested in triplicate 200  $\mu$ l reactions containing 100  $\mu$ l of diluted growth medium (or a PBS control) and 2 to 10  $\mu$ l HRP-CPI2-Trisacryl beads. The mixture was incubated in a 96-well filter plate for 10 minutes at room temperature then centrifuged into a catch plate to stop the reaction. 100  $\mu$ l ABTS substrate (2,2-Azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid), US Biological, Swampscott, MA) was then added to each well and the increase in  $A_{405}$  was monitored for 10 minutes. The rate of color development, signifying free HRP which is a measure of the proteolytic activity, was determined for each well and the triplicate values were used to calculate average and standard deviation from the mean.



### Protease assay with dual affinity tag-labeled CPI2S-Trisacryl beads

Alternatively, dual affinity tag released by proteolysis was bound to streptavidin microtiter plates and then detected with nitrilotriacetic acid (NTA) labeled HRP as described by the manufacturer (Pierce Rockland, MD). Briefly, reactions were performed in a 150 µl volume in a 96-well filter plate. Each reaction consisted of 100 µl of CFGM or protease standard and 3.6 mg dual affinity labeled-CPI2S Trisacryl beads. After incubation for 30 minutes at room temperature, the 96-well filter plate was centrifuged into a 96-well plate with streptavidin-coated wells (Reactibind High Binding, Pierce). Binding was allowed for 60 minutes at room temperature with agitation and wells were rinsed three times with TBS-T (TBS buffer with 0.05% Tween 20 (polyoxyethylene sorbitane monolaureate)) for 5 minutes each at room temperature with agitation. Detection was achieved by incubation with 100 µl of 1:1000 nitrilotriacetic acid (NTA)-labeled HRP (Qiagen) in TBS-T for 60 minutes at room temperature with agitation after which the Ni<sup>2+</sup>-NTA-HRP was removed and the wells washed three times with TBS-T as described above. 100 µl of 3,3',5,5' tetramethyl benzidine (TMB substrate SureBlue, KPL) was added and immediately transferred to a plate spectrophotometer to monitor the development of color at 650 nm for 5 minutes. The rate of color development (in mOD A<sub>650</sub>/min, Vmax) was determined.

### Purification of proteases able to clip CPI2

Bacteria were grown overnight in 25 ml cultures using the conditions described above and proteases were isolated from CFGM. Samples were kept at 4 °C during all purification steps. 80% ammonium sulfate (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was added to the CFGM and the precipitated proteins were collected by centrifugation at 13,000 rpm for 20 min then solubilized in column buffer (50 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and 10 mM Tris-Cl pH 8.0). Gel filtration chromatography was performed with a Superdex 75 10/300 GL column (GE Healthcare Bioscience Corp, Piscataway NJ) equilibrated with column buffer. The protein concentration (A<sub>280</sub>) of the column eluant was continuously monitored and the protease activity within each 1 ml fraction was tested by combining 20 µl with 20 µl of rinsed HRP-CPI2-

Trisacryl beads for 5 minutes. Fractions with significant protease activity were pooled, brought to a final concentration of 2.1 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, and clarified by centrifugation for 7 min at 7,300 rpm. Pooled fractions (typically 7 to 8 ml) were subject to hydrophobic interaction chromatography using a phenyl Hi Trap column (GE Healthcare Bioscience Corp.) equilibrated with 50 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> in 10mM Tris-Cl pH 8.0. Proteins bound on the column were eluted with a gradient from 2.0 to 0.05 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> in 10mM Tris-Cl pH 8.0. The conductivity, protein content (A<sub>280</sub>), and CPI2 proteolytic activity of each collected fraction was monitored.

N-terminal protein sequencing

Purified proteases were precipitated by adding 1:10 volume of trichloroacetic acid, incubating on ice for 30 min, then centrifugating for 10 min at 7,300 rpm. The precipitate was washed with 500 µl ice-cold acetone then suspended in 30 µl protein sample buffer (Laemmli Sample Buffer) and heated to 100°C for 10 min. Proteins were separated by SDS-PAGE using a 15% gel and transferred to PVDF membrane (Millipore Immobilon). Proteins were visualized with 0.05% Coomassie blue and excised proteins were sent for N-terminal sequencing at either Commonwealth Biotechnologies Inc. (Richmond, VA) or Proteos (Kalamazoo, MI). The N-terminal peptide sequences were evaluated for homology to proteins in Genbank and those with the highest degree of identity are reported.

Cleavage site mapping

Unlabeled CPI2 (5 µg) was incubated in a 100 µl reaction with buffer, 1 µg/ml human neutrophil elastase, 10 ng of *S. aureus* GluV8, 50 ng of *P. aeruginosa* LasB, or cell-free growth medium from overnight cultures of *S. aureus*, *S. pyogenes*, *E. faecalis*, *P. mirabilis*, or *P. aeruginosa* diluted to the equivalent of 1x10<sup>7</sup> CFU/ml. Each reaction was incubated for 60 minutes at room temperature then flash frozen. A portion of each sample was then subject to high sensitivity nanospray mass spectroscopy using a Thermo Finnigan LTQ ion trap

mass spectrometer (Tufts University Core Facility, Boston, MA). Prominent peptide fragments were identified by comparison of spectra to a buffer-treated control. Cleavage sites were determined by matching the mass of an actual fragment to those possible at each cleavage site.

### Prevalence of protease-encoding genes in wound isolates

The presence of the gene encoding the CPI2-reactive protease identified from purification and N-terminal sequencing was determined by gene amplification using the polymerase chain reaction (PCR). Genomic DNA was extracted and purified from colonies of each wound isolate using the MasterPure Gram Positive DNA Purification Kit (Epicentre, Madison, WI) following manufacturer's instructions. A typical 50 µl PCR reaction consisted of 2 µl of genomic DNA, 5 µl of 10X Thermapol buffer, 1 µl of 10 mM dNTPs, 0.5 µl of a 100 µM stock of each primer, and 0.5 µl Taq polymerase. The samples were heated at 94 °C for 3 minutes then subject to 25 cycles of 92 °C for 30s, denaturing temperature (55 °C for *P. mirabilis*, 57 °C for *E. faecalis*, 61 °C for *S. pyogenes* and *P. aeruginosa*, and 65 °C for *S. aureus*) for 30 seconds, and 72 °C for 30 seconds with a final extension at 72 °C for 5 minutes. Primer pairs used for PCR, listed 5' to 3' for forward and reverse, were:

*E. faecalis gelE*, GGTACAGGCATCTTTGTTGG and

CTTGATCAACTGGATTTCCTG

*P. aeruginosa lasB*, GGAATGAACGAAGCGTTCTC and

GGTCCAGTAGTAGCGGTTGG

*S. aureus sspA*, GTTATATTACCAAATAACG and GCTAAATCACCTTCGCCTG

*S. pyogenes speB*, GGTATCAGATTATTAAGTC and

CCGTTAGCGTCAAATGATCC

*P. mirabilis zapA*, CCGCAGGAAAACATATAGC and CAGAAGCCTTCATTCC

PCR products were analyzed by agarose gel electrophoresis and strains whose DNA generated a product of the correct size were considered to contain the protease gene.

*Clinical study design*

Patient selection

The study population included patients with wounds of mixed etiology that had not healed in 60 days who provided written consent to be enrolled in the study. A university hospital wound care clinic (UMASS Medical School) served as the study setting. The study protocol was approved by the institutional review board (IRB) prior to the enrollment of subjects. Patients were screened and selected for the study based on the following criteria: at least 18 years of age, non-healing wound for 60 days, questionable infection status (i.e. not obviously infected or uninfected), not treated with an enzymatic debriding agent in the last 30 days, not receiving any investigational therapy in the last 60 days, not known to be HIV positive, and no malignancy or co-existing condition that would increase patient risk. Eligible patients were invited to enter the study, and informed consent was obtained from the subject or their legal representative.

Sample collection and extraction

Wound tissue fluid was gathered specifically for the purpose of this study during the course of a regularly scheduled clinic appointment. Wound tissue fluid was collected from viable tissue within the wound bed using a sterile Dacron swab housed in a collection tube. If indicated in a course of treatment, non-viable tissue and cellular debris were removed by sharp debridement, surgical debridement, or cleansing of the wound with sterile saline. Tissue fluid was extracted from the wound bed using the Levine quantitative collection technique: rotating the swab over a 1 cm<sup>2</sup> area of viable tissue with sufficient pressure to express fluid (29). The swab was then returned to the collection tube and processed within 20 minutes of sampling.

Swabs were weighed in order to estimate the volume of tissue fluid collected (difference in weight relative to the average weight of a dry swab). Tissue fluid was extracted from the swab by agitation in 1.0 ml of extraction buffer (DPBS) for 30 seconds. An aliquot of the sample (0.4 ml) was placed in a

sterile tube and stored at 4°C prior to quantitative microbiological analysis. The remaining 0.6 ml was flash frozen in liquid nitrogen and stored at -80°C.

### Quantitative microbiology

A 100 µl aliquot of each swab extract was diluted  $10^{-2}$  and  $10^{-4}$  in PBS and then plated onto tryptic soy agar with 5% sheep's blood, MacConkey agar, and chocolate agar (PML Microbiologicals Inc., Wilsonville, OR). The plates were incubated for 48 hours at 37°C and the number of colonies of each distinct strain was counted. The identity of each strain was determined using the Vitek II system supplemented by standard biochemical assays when needed. These studies were independently performed by the UMass Memorial Medical Center Clinical Microbiology Laboratory.

### Treatment and assay of clinical samples

Samples were thawed on ice and 400 µl of the sample was mixed with and passed through 500 µl of a 50% slurry of sulfopropyl resin (SP; GE Healthcare) by centrifugation in a microfuge spin filter. This treatment was found to selectively remove the host enzyme, human neutrophil elastase (HNE) from the tissue fluid sample (see Results Section). The bacterial protease activity in the filtrate, referred to as SP-treated sample, was measured using dual affinity labeled-CPI2S microbeads as described below. Samples were assessed in groups of 10 with controls that included buffer only (negative control) and 0.1 µg/ml *S. aureus* GluV8 protease (positive control) (Worthington Biochemical). Serial dilutions of the dual tag CPI2S peptide were also run on every assay plate to generate a standard curve.

Human neutrophil elastase activity in SP-treated and untreated samples was tested using fluorogenic elastase substrate V (Methoxysuccinyl-ala-ala-pro-val-7-amino-4-methylcoumarin, Calbiochem). Proteases in bacterial cell-free growth medium from *S. aureus*, *E. faecalis*, *S. pyogenes*, *P. aeruginosa*, or *P. mirabilis* did not significantly cleave this substrate (data not shown). A HNE

standard curve was constructed using serial 1:2 dilutions of 100 µg/ml human neutrophil elastase (Athena).

The total protein concentration in tissue fluid samples was determined by a Bradford assay (BioRad, Hercules, CA). A standard curve generated with gamma globulin standards (0 to 300 µg/ml) was used to determine the concentration (mg/ml).

Protease assay with dual affinity tag-labeled CPI2S-Trisacryl beads

Reactions were performed in a 150 µl volume in a 96-well filter plate. Each reaction consisted of 120 µl of extracted wound fluid sample, 20 µl dual affinity labeled-CPI2S Trisacryl beads from a 180 mg/ml stock, and 10 µl of assay buffer (DPBS plus 0.1% PEG 5000). After incubation for 30 minutes at room temperature, the 96-well filter plate was centrifuged so that the filtrate was collected in a 96-well plate with streptavidin-coated wells (Reactibind High Binding, Pierce). Binding was allowed for 60 minutes at room temperature with agitation and wells were rinsed three times with TBS-T (TBS buffer with 0.05% Tween 20 (polyoxyethylene sorbitane monolaureate)) for 5 minutes each at room temperature with agitation. Detection was achieved by incubation with 100 µl of 1:1000 nitrilotriacetic acid (NTA)-labeled HRP (Qiagen) in TBS-T for 60 minutes at room temperature with agitation after which the Ni<sup>2+</sup>-NTA-HRP was removed and the wells washed three times with TBS-T as described above. An aliquot (100 µl) of 3,3',5,5' tetramethyl benzidine (TMB) substrate (SureBlue, KPL) was then added to each well and the ELISA plate was immediately transferred to a plate spectrophotometer to monitor the development of color at 650 nm for 5 minutes. The rate of color development (in milliOD units A<sub>650</sub> per minute) was determined from a linear portion of the data. This value was converted to the mass of clipped peptide produced (ng/ml) through use of a standard curve run on every plate.

## RESULTS

### Detection of bacterial proteases *in vitro* using the CPI2 peptide

To develop a broad-spectrum assay, a peptide substrate capable of detection of proteases produced by a number of different bacteria was needed. We utilized a peptide derived from the reactive site loop (RSL) of the mammalian protease inhibitor  $\alpha$ 1-antitrypsin ( $\alpha$ 1AT) since native  $\alpha$ 1AT has been demonstrated to be cleaved by proteases from *P. aeruginosa*, *S. aureus*, *S. marcescens*, *S. pyogenes*, and *P. gingivalis* in that region (30). A 13 amino acid polypeptide derived from the  $\alpha$ 1AT RSL was initially tested as a protease substrate. This peptide (called CPI2), corresponding to amino acids 349 to 361 of the human  $\alpha$ 1AT RSL, was synthesized with an C-terminal lysine or cysteine GAMFLEAIPMSIPK or GAMFLEAIPMSIPC to allow labeling with a dual affinity tag (biotin and polyhistidine) or with HRP enabling measurement of cleavage. Shorter versions of this peptide (called CPI2S, GMAFLEAIP) were shown to have a reduced sensitivity to human neutrophil elastase (HNE) (see below).

### Microbead protease assay format

The CPI2 (or CPI2S) peptide was incorporated into a novel microbead-based assay format designed to improve the speed and sensitivity of bacterial protease detection (Figure 1). The main purpose of this format is to allow simple and rapid separation of clipped from unclipped peptide substrate so that the amount of clipped peptide could be measured via the tag reporter. CPI2-HRP or CPI2-Biotin-polyhistidine was attached to a chromatography microbead anchor by covalent conjugation of the available N-terminus of the peptide to carboxyl methyl groups on the Trisacryl CM bead surface. In this configuration, CPI2 serves as a hydrolyzable tether that attaches the reporter to the microbead. Bacterial protease cleavage of CPI2 should release the reporter from the surface of the microbead so that the free tag can readily be separated from the microbead-bound tag by centrifugation or filtration. The color change is measured by isolating free HRP (or indirectly through NTA-HRP) using a

colorimetric substrate that correlates to the amount of peptide clipped and the activity of bacterial protease in the test sample.

To verify the ability of CPI2S-Biotin-polyhistidine microbeads to measure bacterial protease activity, CFGM from five bacterial cultures was diluted to  $1 \times 10^8$  CFU/ml and mixed with the bead conjugate for 30 minutes at room temperature, the beads were removed by filtration, and the free tag in the filtrate was measured. As seen in Figure 2A, each bacterial protease preparation released the detectable dual affinity tag while media and buffer controls lacking protease did not release a significant amount of reporter. This result is a clear demonstration that tethering CPI2S between an affinity tag and a polymer microbead did not alter the ability of bacterial proteases to clip the peptide. The release of the tag was dependent on pathogen concentration (CFU/ml), as shown by testing dilutions of CFGM from overnight cultures of bacterial wound isolates with HRP-CPI2-microbeads. The initial concentration of the bacterial culture was determined by optical density, the cells were removed, and the resulting CFGM was diluted to concentrations between  $1 \times 10^8$  to  $1 \times 10^6$  CFU/ml in fresh medium. Since the *S. pyogenes* CFGM contained much higher protease activity than the other preparations, the equivalent of  $1 \times 10^7$  to  $1 \times 10^5$  CFU/ml was tested for this species and the released HRP was diluted prior to measurement. In a five-minute assay, the bacterial concentration of the sample correlated to the rate of colorimetric substrate hydrolysis (Figure 2B). *S. pyogenes* CFGM had roughly 10-fold more activity (taking the HRP dilution into account) than *P. aeruginosa*, which was next most active CFGM. The remaining bacterial species, *S. aureus*, *E. faecalis*, and *P. mirabilis*, produced roughly equal proteolytic activity in this assay. This data clearly indicates that the response of the HRP-CPI2-microbeads is dependent on the amount of protease within the sample. It also shows that bacterial proteases produced by  $1 \times 10^6$  CFU/ml of bacteria or fewer can be effectively detected in 5 minutes.

We examined the limit of detection using HRP-CPI2-microbeads with several different wound isolates. Since a threshold level of  $10^5$  CFU bacteria per gram of tissue is considered to affect healing, CFGM from wound isolates grown



overnight was diluted to an *in vitro* equivalent above the threshold,  $10^6$  CFU/ml, or below the threshold,  $10^4$  CFU/ml. CFGM from each wound isolate adjusted to  $10^6$  CFU/ml generated a signal that was significantly above that of a buffer control in a 15-minute assay (Figure 3). At  $10^4$  CFU/ml only CFGM from *S. pyogenes* and *P. mirabilis* generated a significant signal, which was consistent with the high level of protease activity seen from  $10^6$  CFU/ml of these species. This indicated that the HRP-CPI2-microbead assay detected bacteria at a level above the threshold level of  $10^5$  CFU/ml in 15 minutes *in vitro*. The protease activity detected was relatively consistent for the isolates within a particular species and there was more of a difference in the average activity between bacterial species. *S. pyogenes* cultures showed the most activity, followed by *P. mirabilis*, *P. aeruginosa*, *S. aureus*, then *E. faecalis*.

#### CPI2 proteolytic activity in bacteria isolated from wounds

The bacterial species demonstrated to produce proteases that cleave CPI2 are some of the more prevalent species isolated from wound infections (30). We therefore, hypothesized that bacterial protease activity toward CPI2 could serve as a marker for bacteria in wounds. To do so, CPI2-cleaving proteases would need to have a high prevalence in wound isolates of these species. This is particularly relevant in light of the demonstrated variability in certain virulence traits among clinical bacterial isolates (31).

To determine the pervasiveness of CPI2-cleaving proteases in bacterial wound isolates, a collection of 20 strains each of *S. aureus*, *E. faecalis*, *S. pyogenes*, and *P. aeruginosa* that were isolated from open acute or chronic wounds was assembled. To ensure diversity in our strain collection, five isolates of each species were gathered from four different medical centers at various locations in the US. Unfortunately, one strain was incorrectly identified as *P. aeruginosa*, resulting in its exclusion from further analysis. Protease production by these clinical wound isolates was assessed and, the vast majority of the wound isolates produced detectable protease activity *in vitro*. The percentage of wound isolates showing CPI2 proteolysis was 90% for *S. aureus*, 80% of *E.*

*faecalis*, 85% of *S. pyogenes*, and 94.7% of *P. aeruginosa*. This was consistent with the prevalence of *in vitro* protease activity seen in isolates of these species from other human infections (20, 26, 27), though somewhat higher than the proteolytic activity prevalence demonstrated previously in *P. aeruginosa* isolated from chronic wounds (26). As evident from the standard deviations in rate of hydrolysis (Table 1), the variation of CPI2/CPI2S-dependent protease activity between strains of a particular species was approximately 6-10%. Nevertheless, this evidence showing that most bacterial strains from wounds produce proteolytic activity toward the CPI2 substrate indicated that proteases could be reliable indicators of wound bacteria. Protease activity appears to be a good candidate for use as markers in a diagnostic test to detect bacteria within wounds.

Identification of bacterial proteases that clip CPI2 and characterization of their target sites

To gain additional information on the proteases that may serve as markers, enzymes from *S. aureus*, *S. pyogenes*, *E. faecalis*, or *P. aeruginosa* that cleave CPI2 were identified. In addition, the protease from *Proteus mirabilis* that clipped CPI2 was identified in order to characterize an additional gram-negative organism. Proteins from the CFGM of wound isolate cultures were precipitated with ammonium sulfate and the protease with significant activity toward CPI2 was then purified using size exclusion and hydrophobic interaction chromatography. After these purification steps, preparations were analyzed by gelatin zymography and active proteases were isolated by SDS-PAGE and subject to N-terminal sequencing. Each purified protein showed significant N-terminal amino acid sequence homology to a known secreted proteolytic enzyme from the corresponding bacterium (Table 2). *P. aeruginosa* elastase (LasB), *S. aureus* GluV8 (SspA), and *S. pyogenes* SpeB had been previously shown to clip the RSL of native  $\alpha$ 1-AP and disrupt its inhibitory activity, so their discovery was not surprising (30). However, these data provided the first evidence that *E.*

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3 *faecalis* gelatinase (GelE) and *P. mirabilis* ZapA are able to target CPI2 and  
4 therefore the RSL of native  $\alpha$ 1-AP.  
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7 To localize the cleavage sites of the identified proteases within CPI2,  
8 unlabeled CPI2 was treated with the CFGM from *S. aureus*, *P. aeruginosa*, *E.*  
9 *faecalis*, *P. mirabilis*, or *S. pyogenes* and the digest was evaluated using mass  
10 spectroscopy. Comparison of the sizes of the peptide fragments in digested  
11 samples with untreated CPI2 enabled the identification of the cleavage products,  
12 and the masses of these products allowed mapping of the target site(s) (Figure  
13 4a). The CPI2 peptide cleavage map for each of the bacterial proteases is  
14 shown in Figure 4b. Untreated CPI2 had a molecular mass of 1501 Da, while the  
15 major digestion products were 1242 Da for *P. aeruginosa*, 853 Da for *S. aureus*,  
16 1095 Da and 1242 Da for *E. faecalis*, and 853, 1101, and 1188 Da for *P.*  
17 *mirabilis*. The primary target site of HNE within CPI2 was also mapped using  
18 mass spectroscopy as described for the bacterial proteases. HNE produced a  
19 CPI2 fragment of 1101 Da indicating that the target site was between Met/Ser  
20 within CPI2, which was in a distinct location from the target sites of most of the  
21 bacterial proteases tested. A schematic representation of the major peptide  
22 fragments for each protease is shown in Figure 4b, similar results were obtained  
23 for *S. aureus* using purified V8 protease and for *P. aeruginosa* using purified  
24 LasB (not shown). Digestion of CPI2 with *S. pyogenes* CFGM produced peptide  
25 fragments of 1106, 1162, and 1278 Da (not shown), which did not correspond to  
26 any of the possible fragments that could result from peptide bond cleavage. One  
27 possible explanation is that an enzyme in *S. pyogenes* CFGM, in addition to  
28 cleavage of the peptide, is capable of modifying the mass of the CPI2 peptide  
29 fragments in another manner. Common posttranslational modifications in *S.*  
30 *pyogenes* include deformylation and the addition of D-alanine or thioether amino  
31 acids such as lanthionine and 3-methylanthionine. Another possibility is that *S.*  
32 *pyogenes* was grown in a complex meat media which may have additional  
33 peptide fragments that could mask the signal.  
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55 As a next step, the prevalence of the identified CPI2-cleaving proteases  
56 from each species was determined. The collection of wound isolates was  
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3 screened for the presence of the species-specific gene encoding the CPI2-  
4 cleaving protease identified: *lasB*, *sspA*, *gelE*, or *speB* and *zapA*. Using  
5 polymerase chain reaction to specifically amplify a fragment of each of the  
6 protease genes from isolates, we determined that in each case the gene was  
7 present 100% of the time as shown in the 1% agarose gel photos in Figure 5.  
8 Therefore, the genes encoding the CPI2-targeting proteases did not appear to be  
9 a variable virulence trait in wound isolates of these species.  
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18 Time Course with V8 Protease

19 In order to appreciate the time dependence of the proteolytic activity for  
20 the CPI2S peptide dual affinity tag labeled beads, we performed a time course  
21 with 50 ng/ml of V8 protease standard which is comparable to the activity of *S.*  
22 *aureus* at 10<sup>5</sup> CFU per ml. Our findings indicate that within 10-15 minutes there  
23 is significant signal above the background of the uninfected media control (Figure  
24 6). For our clinical studies reported below we conservatively used the time point  
25 of 30 minutes to measure the protease activity of actual wound fluids.  
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33 The ability of host proteases to process CPI2

34 A number of research studies have demonstrated that the concentrations  
35 of neutrophil proteases such as human neutrophil elastase (HNE) and  
36 gelatinase-type matrix metalloproteinase-2 and -9 (MMP-2 and MMP-9) are  
37 elevated in chronic wounds compared to acute wounds (37-38). Since HNE is  
38 the primary target of  $\alpha$ 1-AP *in vivo* and both HNE and MMP-2 and -9 are known  
39 to cleave the reactive site loop of native  $\alpha$ 1-AP (32-34), we investigated the  
40 ability of these proteases to clip the CPI2 peptide. Commercial preparations of  
41 HNE or activated MMP-2 or MMP-9 were diluted to physiologically elevated  
42 levels and analyzed in the protease assays. The increase in Vmax signal seen  
43 with HNE demonstrated that this protease was able to efficiently clip CPI2  
44 comparable to the V8 bacterial protease control (Figure 7). In contrast, the signal  
45 for HNE with the shorter version CPI2S produced a signal that was much  
46 reduced without affecting the activity of V8 protease. No significant activity was  
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seen with MMP-2 or MMP-9 with either peptide suggesting that none of the residues in CPI2 are sensitive to MMPs (not shown).

In Table 1, a direct comparison of the amount of HRP released from CPI2S and CPI2 HRP-beads by bacterial proteases present in CFGM shows that the bacterial proteolytic activities are retained while dramatically reducing the sensitivity to HNE. In particular, proteases from *P. aeruginosa* or *S. pyogenes* released similar amounts of HRP from CPI2 and CPI2S HRP-microbeads. *S. aureus* and *E. faecalis* proteases were more active toward HRP-CPI2-microbeads than HRP-CPI2S-microbeads, but the ratio of activity from bacterial proteases and HNE showed that CPI2S appears much more selective for bacterial proteases than CPI2. The bacterial protease-to-HNE activity ratios reflect that CPI2S is much less sensitive to HNE relative to each bacterial protease. Thus the reduced ability of HNE to clip CPI2S was not accompanied by a corresponding reduction in bacterial protease activity. This truncation of the peptide substrate therefore improved the specificity of detection of bacterial proteases.

### Clinical study results

In the clinical study at UMASS Medical School, the 100 chronic wounds had mixed etiologies: pressure ulcers, diabetic foot ulcers, venous ulcers, and a small proportion of post-surgical and post trauma non-healing wounds (Table 3). Sharp debridement was performed on wounds from 17 patients, while 12 wounds were debrided surgically prior to wound fluid collection. The remaining wounds were cleansed prior to sampling. Fluid collection was performed with a Dacron swab using the Levine technique (29). After collection, wound fluid was extracted from the swab into buffer (DPBS) and a portion of the extract was used for enumeration and identification of the aerobic bacteria using standard culture techniques. There were 238 bacterial strains isolated from 89 patient samples, 11 samples yielded no bacteria. The clinical samples had a preponderance of *Staphylococcus aureus* and there was an average of 2.4 +/- 1.5 bacterial species per wound falling into the range typical of other microbiological analyses (35).

The 15 bacteria most frequently isolated from the clinical samples are indicated in Table 4.

The standard curve for HNE was performed with a fluorogenic elastase substrate V (Methoxysuccinyl-ala-ala-pro-val-7-amino-4-methylcoumarin) in triplicate. The range of the elastase concentration in the swab samples collected using the Levine procedure was found to be 0-25.4 µg/ml, with the mean HNE concentration in 100 samples being  $0.56 \pm 0.26$  µg /ml and standard deviation (SD) being 2.63 µg /ml. Although only a few wound fluid samples were found to possess elastase at concentrations high enough to match the bacterial proteases activity in terms of interaction with a CPI2 substrate (see Figure 8), and although using CPI2s substantially suppresses HNE-substrate interaction, we chose to find additional methods to remove HNE from the diagnostic test so that it would not interfere with the assay. HNE has a very basic isoelectric point of 8.77 and we determined that a cationic exchange chromatography resin such as sulfopropyl (SP) would remove >80% of the HNE. The range for HNE concentration in SP-treated samples became 0 to 1.6 µg /ml, with the mean value being  $0.07 \pm 0.02$  µg /ml and SD=0.22 µg /ml.

In addition to testing HNE activities, we measured the protease activity of each clinical sample. Each reaction consisted of 120 µl of extracted wound fluid sample, 20 µl of dual affinity labeled-CPI2S Trisacryl beads from a 180 mg/ml stock, and 10 µl of assay buffer (DPBS plus 0.1% PEG 5000). After centrifugation, the detection of released dual affinity tag was performed in a streptavidin ELISA plate as described in the methods section. The data collected was a measurement of protease activity in each of the samples (mOD/min) which was then converted to total nanograms of peptide cleaved using a standard curve of CPI2S dilutions. A subsequent analysis found no apparent correlation between the level of HNE and the amount of CPI2S clipped peptide in SP-treated samples ( $R^2=5 \times 10^{-3}$ ,  $F=0.53$ ,  $p=0.47$ ). Thus, we conclude that our detection technique is specific for bacterial proteases.

The microbiology results were obtained from the UMASS Medical School Clinical Microbiology Lab and the total bioburden (CFU/ml) was compared to the

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3 protease activity/nanograms of peptide clipped for each sample. Statistical  
4 analysis of the clinical data demonstrated a significant correlation between  
5 proteolytic activity and total bioburden ( $p < 10^{-4}$ ).  
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9 A threshold level of bioburden to discriminate between infected and  
10 uninfected wounds is believed to be in a range of  $10^5$  -  $10^6$  CFU/ml (1, 12). The  
11 Wound Healing Society recommends that all chronic and acute wounds should  
12 be treated at a bioburden level of  $\geq 1 \times 10^6$  CFU/ml for most bacteria. In our  
13 attempt to reliably distinguish between infected and uninfected wounds on the  
14 grounds of exhibited proteolytic activity, we built a family of Receiver Operating  
15 Characteristics (ROC) that gave a relationship between sensitivity and false  
16 positive rate of detection for every particular choice of bioburden threshold  
17 discriminating between infected and uninfected wounds. The greater the area  
18 under the ROC curve (0.5 corresponds to a test of no diagnostic value, while 1 is  
19 equivalent to a golden standard), the more robust a diagnostic test is believed to  
20 be. The choice of bioburden cutoff at  $3 \times 10^5$  CFU/ml yields an optimal ROC to  
21 maximize an area under the curve (AUC), with the bootstrap estimate for AUC  
22 being  $0.84 \pm 0.06$  (SAS version 9.0, SAS Institute Inc., Cary, NC). Similarly, the  
23 Leave-one-Out method of cross-validation yields  $AUC = 0.84 \pm 0.05$  ( $\chi^2 = 267.7$ ,  
24  $p < 10^{-4}$ ), while the most conservative method of Split-Sample validation results in  
25  $AUC = 0.84 \pm 0.09$  ( $\chi^2 = 85.6$ ,  $p < 10^{-4}$ ). The mean activity of uninfected samples  
26 (bioburden  $< 3 \times 10^5$  CFU/ml) is found to be  $26.9 \pm 8.7$  ng of peptide clipped per  
27 sample, which is much lower than the mean activity of infected samples  
28 (bioburden  $\geq 3 \times 10^5$  CFU/ml) found to be  $77.1 \pm 6.2$  ng of clipped peptide. The  
29 comparison of the mean proteolytic activity of infected and non-infected clinical  
30 samples (Figure 9) proves their significant difference, with a Wilcoxon rank-sum  
31 test (equivalent to the Mann-Whitney U-test) giving us the two-tailed asymptotic  
32 p-value for the probability of the difference occurring by chance alone as  $p < 10^{-4}$ .  
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36 We found that limiting the collection volume to 0.1 ml of wound fluid,  
37 suppresses sample-to-sample variability and helps us to better distinguish  
38 between infected and uninfected wounds in terms of protease activity.  
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The choice of activity cutoff in the range from 33 ng to 50 ng of peptide clipped/0.1 ml yields detection sensitivity of 89.6% and a specificity of 69.6% relative to the reference standard of quantitative microbiology (Figure 10). When discriminating between “infected” and “uninfected” wounds regarding the level of their activity measured per 0.1 ml of sample, a Wilcoxon rank-sum test gives us the two-tailed exact p-value for the probability of rejecting the null hypothesis by mistake as  $p=6.78\times10^{-8}$ .

In Fig. 11 (a) we sorted all 100 wounds according to their level of bacterial protease activity (below/above 45 ng of clip per 0.1 ml) and according to their bioburden (below/above  $3\times10^5$  CFU/ml). There is a two orders of magnitude difference in mean activity values between “active” and “inactive” groups vs. only 5 times difference in mean activity values between “infected” and “uninfected” groups.

We speculate that some “false positives” of detection (high level of protease activity measured in wounds with a low bacterial load) come from intrinsic enzymatic activity associated with a dead tissue, present in non-debrided wounds. While bacteria are mainly immobilized within the dead tissue, the enzymes they produced are mobile and able to contribute to the net protease activity found in the wound fluid. The analysis of data obtained from all 29 debrided wounds in our study is depicted in Fig. 11b.

Whereas a difference in the mean activity values between “active” and “inactive” groups stays approximately the same, the difference in the mean activity values between “infected” and “uninfected” groups increases substantially. In terms of ROC analysis, an estimate for the area under the curve gives much larger  $AUC=0.92\pm0.05$ . The same choice of activity threshold (within a range of 33 to 50 ng/0.1 ml) yields marginally better sensitivity of 92.9 % but much better specificity of 80%. If specificity of detection is of great concern (a low prevalence case), it can be increased even more at the penalty of lower sensitivity, namely, setting activity threshold in the range of 100 to 130 ng /0.1 ml results in 85.7% sensitivity and 86.7 % specificity of detection.



## DISCUSSION

The risk of wound infection depends on a combination of bacterial and patient factors. Patient factors include the underlying disease state, immune status, and local influences: wound location, size, depth, oxygen perfusion, and the presence of foreign material. Bacterial factors include the bacterial concentration, the number of bacterial species and the virulence potential of microbes, bacterial biofilms, and synergistic interactions (36). Despite this basic understanding, there is relatively little information regarding the virulence mechanisms that assist bacteria in causing wound infection. The data presented here indicate that proteolytic enzyme production is a common virulence trait among bacterial wound isolates. Consequently, bacterial protease activity has the potential to be a diagnostic marker for the detection of bacteria in wounds.

The first goal of this study was to identify a peptide substrate able to simultaneously detect proteases produced by the different bacterial species found in wounds. We demonstrated that CPI2, a peptide substrate that is the reactive site loop (RSL) of the wide spectrum protease inhibitor human alpha-1 antitrypsin ( $\alpha$ 1AT), was clipped by secreted proteases from five predominant bacterial species prevalent in wounds, namely *S. aureus*, *E. faecalis*, *S. pyogenes*, *P. aeruginosa*, and *P. mirabilis*. In similar studies, CFGM from overnight cultures of *Serratia marcescens*, *Enterobacter cloacae*, *Morganella morganii*, *Acinetobacter baumannii*, and *Stenotrophomonas maltophilia* also clipped the CPI2 peptide (data not shown). This led to the conclusion that CPI2 is a capable broad-spectrum substrate for bacterial proteases. This was consistent with a previous report of a multi-step assay used to detect protease activity in unknown samples based on the broad susceptibility of  $\alpha$ 1AT to proteolytic attack. One major improvement over that report is the use of a target peptide representative of the  $\alpha$ 1AT RSL, rather than the whole protein, and its incorporation into a simple, rapid assay format.

Purification of the primary proteases that target CPI2 and mapping of their target sites demonstrated that CPI2 contains a number of residues surrounding each of the identified bacterial protease target sites. This includes at a minimum

the P'2, P'1, P1, and P2 residues, which gives bacterial proteases adequate spacing to recognize the target and efficiently clip the peptide. The identified target site for *S. aureus* GluV8 in this study is identical to that determined previously for native  $\alpha$ 1AT (37) and consistent with the P1' glutamic acid preference of GluV. In contrast, the mapped site for *P. aeruginosa* LasB cleavage, Met/Phe, is different than that previously reported for native  $\alpha$ 1AT. Morihara et al. (1979) previously identified Pro/Met as the LasB cleavage site (38). This discrepancy may be due to the different context of the target site within the soluble CPI2 peptide and within the RSL in the  $\alpha$ 1AT protein, which could alter the preferred site of cleavage.

Another important goal of this study was to examine the prevalence of protease production by bacterial wound isolates. The proteases identified to clip CPI2 were shown to be invariable virulence traits in 20 wound isolates of *S. aureus*, *E. faecalis*, and *S. pyogenes*, 19 wound isolates of *P. aeruginosa* and 3 wound isolates of *P. mirabilis* from four clinical locations within the US. This was demonstrated by species-specific PCR amplification indicating that each wound isolate contained the gene of interest. When expression of these genes was determined by measuring CPI2-dependent protease activity in the wound isolates, >80 to 95% produced detectable amounts of protease *in vitro*. In addition, 3 of 3 *P. mirabilis* wound isolates displayed activity (data not shown). The differences evident in protease activity among species and between species are most likely due to variation in the protease expression levels that result in a different concentration of proteases in the CFGM from each isolate. Also, each bacterial enzyme may have a different specific activity towards CPI2.

Another significant aspect of this work is the demonstration of a simple, colorimetric assay able to provide a rapid indication of protease activity. The primary reagent for this assay is the reporter-peptide substrate-microbead conjugate produced by covalent attachment of a peptide labeled with an affinity tag to the modified surface of a polymer bead (Figure 1). This configuration allows simple and rapid separation of reporters released from beads due to peptide cleavage and those that remain on the bead attached to unclipped

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3 peptide. Similar constructs have been used with peptide microarrays to study  
4 protease catalytic activity and cross-reactivity though these mostly utilize a  
5 fluorescent reporter. The major potential concern with this format, access of the  
6 peptide tethered-bead substrate to bacterial proteases, does not appear to be a  
7 significant issue since CFGM from multiple species clipped CPI2 and CPI2S and  
8 released detectable HRP or the dual affinity tag (Figure 2a+b). Since this  
9 microbead format could be used with virtually any peptide substrate, the assay  
10 could be used to rapidly measure any protease activity with a specificity  
11 determined by the peptide substrate. Also, our current approach produces a  
12 colorimetric readout, replacement of the affinity tag with another reporter, such as  
13 a fluorescent or colored dye would allow other types of output. Consequently,  
14 the microbead-based assay could have utility in a number of laboratory or clinical  
15 diagnostic applications.

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17 The specificity of the HRP-CPI2-microbead assay for bacterial proteases  
18 is critical for a clinically useful test to detect bacteria in wound samples. The  
19 CPI2 peptide is susceptible to cleavage by the inflammatory protease HNE but  
20 not MMP9, which can be present at high levels in a subset of chronic, or non-  
21 healing, wounds (39). Since elevated HNE could cause protease activity to be  
22 detected in the absence of bacterial proteases, CPI2 was altered to reduce  
23 cleavage by HNE relative to bacterial protease. Through peptide mapping of the  
24 target sites of bacterial proteases and HNE within CPI2 (Figure 4a+b), four C-  
25 terminal amino acids that contained the HNE target site were removed to  
26 generate CPI2S, while the N-terminal portion of the peptide containing the  
27 bacterial protease target sites was left intact. This dramatically improved the  
28 bacterial specificity of the peptide substrate without significantly raising the limit  
29 of detection. CPI2S showed a 93% reduction in cleavage by HNE and an  
30 increased selectivity (represented by bacteria protease-to-HNE ratio) toward  
31 bacterial proteases. This new peptide substrate, in addition to a sample filtration  
32 through the SP-resin to selectively bind and reduce HNE content, was used to  
33 detect bacterial protease activity in specimens from wounds and correlate this  
34 activity to bacterial content.

We found that bacterial protease activity in samples from chronic wounds correlates well with the concentration of bacteria (bioburden) found in the wound. An initial 100 patient clinical study suggested that our assay can have a sensitivity of about 90% and a specificity of about 70% for detection of wound bioburden exceeding the threshold between  $10^5$  and  $10^6$  CFU/ml. We believe one source of false positives compromising specificity of detection to be an enzymatic activity associated with a dead tissue. Actually, the Wound Healing Society recommends wound debridement to be performed, when necrotic tissue is present. For debrided wounds, our assay demonstrated better performance, with a sensitivity close to 93% and a specificity of 80%. If specificity of detection is of primary concern, it can be increased by adjusting the assay detection threshold, at the cost of lower sensitivity, to yield approximately 86% sensitivity and 87% specificity of detection.

In summary, the studies presented here demonstrate the validity of using bacterial protease activity as a biomarker for the detection of wound bacteria. These enzymes are the first biochemical marker to serve this purpose and the creation of an assay to rapidly and simply measure their activity is a critical step in the development of a rapid test that will help establish an accurate point-of-care infection diagnosis and treatment plan for patients with wounds. A marker such as protease activity depends on both the concentration of bacteria and their ability to express and secrete proteases. Thus protease activity has the ability to provide an indication of both the level and virulence potential of bacteria within wounds and could offer an improved measure of infection status compared to the current standard of bacterial concentration alone. The ultimate goal of this project is to compare the correlation of bacterial protease activity and bacterial concentration to healing outcomes.

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For Peer Review

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**Table 1:** Comparison of protease cleavage of CPI2S and CPI2

	# of Strains with Activity	CPI2 Activity Vmax (OD <sub>405nm</sub> )	CPI2S Activity Vmax (OD <sub>405nm</sub> )	bact/HNE ratio CPI2	bact/HNE ratio CPI2S
<b>HNE</b>	NA	106 +/- 6.6	6.9 +/- 1.8	n/a	n/a
<b><i>S. aureus</i></b>	18/20 (90%)	188 +/- 20	82.9 +/- 5.0	1.8	12.1
<b><i>E. faecalis</i></b>	16/20 (80%)	80.7 +/- 7.5	44.9 +/- 0.9	0.8	6.5
<b><i>S. pyogenes</i></b>	17/20 (85%)	25.7 +/- 2.5	18.8 +/- 0.9	0.2	2.7
<b><i>P. aeruginosa</i></b>	18/19 (94.7%)	194 +/-13.4	152 +/- 31.1	1.8	22.2

**Table 2:** Identification of bacterial proteases able to cleave CPI2

	comparison	match
<i>P. aeruginosa</i>	AEAGGPGGNQ           AEAGGPGGNQ	elastase (LasB)
<i>S. aureus</i>	VILPNNDRHQ           VILPNNDRHQ	glutamyl endopeptidase (GluV8, SspA)
<i>S. pyogenes</i>	GTAEIKQPVVKSLLDS           GTAEIKQPVVKSLLDS	streptopain (SpeB)
<i>E. faecalis</i>	VGSEVTLKNS   *         VASEVTLKNS	coccolysin (GelE)
<i>P. mirabilis</i>	NYSTKVLP     *       NYGTKVLP	mirabilysin (ZapA)

**Table 3:** Chronic Wound Etiology

Wound type, by clinical diagnosis:

- 21 pressure ulcers
- 20 diabetic foot ulcers
- 17 venous ulcers, some patients also diabetic
- 29 etiology not indicated/not known
- 13 post-surgical or post-trauma non-healing wounds

**Table 4:** Composition of Bacteria Found in Chronic Wounds

	#	% of isolates	% of patients
<i>Staphylococcus aureus</i>	43	18.1	43
<i>Corynebacterium spp.</i>	41	17.2	41
<i>Staphylococcus spp. (not aureus)</i>	32	13.4	32
<i>Enterococcus species</i>	16	6.7	16
<i>Pseudomonas aeruginosa</i>	14	5.9	14
<i>Proteus mirabilis</i>	11	4.6	11
<i>Escherichia coli</i>	9	3.8	9
Beta-hemolytic Strep Group B	8	3.4	8
Beta-hemolytic Strep Group G	8	3.4	8
<i>Acinetobacter baumannii</i>	7	2.9	7
<i>Viridans streptococci</i>	6	2.5	6
<i>Klebsiella pneumoniae</i>	6	2.5	6
<i>Morganella morganii</i>	5	2.1	5
<i>Stenotrophomonas maltophilia</i>	5	2.1	5
<i>Klebsiella oxytoca</i>	4	1.7	4

## FIGURE LEGENDS

**Figure 1:** Diagram of the microbead format for measuring protease activity. The CPI2 peptide is covalently conjugated to a Trisacryl microbead (roughly 70  $\mu\text{m}$  in size) via the N-terminus and to a tag or dual tag via a C-terminal cysteine. The peptide acts as a tether that upon cleavage by bacterial proteases releases the detectable tag from the microbead. Free cleaved tag can then be separated from tags that are bound to microbeads by filtration or centrifugation and measured directly or using a colorimetric substrate.

**Figure 2:** *In vitro* measurement of bacterial protease activity using biotin-polyhistidine tagged CPI2 microbeads and HRP-CPI2-microbeads.

A) Demonstration of efficient processing by bacterial proteases. CFGM from cultures of *S. aureus*, *E. faecalis*, *S. pyogenes*, *P. mirabilis*, or *P. aeruginosa* adjusted to  $10^8$  cfu/ml or a control sample (PBS). The media from all the bacteria grow ups (4 different types) was tested as controls and gave the same signal as PBS. Data is not shown to simplify the graph. These samples were tested on Biotin-polyhistidine-CPI2S beads with a 30-minute cleavage time. The beads were removed by centrifugation and free dual tag was measured in an ELISA assay using streptavidin -coated plates and NTA-HRP. Signal was measured by monitoring the increase in color ( $A_{650\text{ nm}}$ ) after addition of TMB substrate.

B) Correlation of signal tag release to bacterial protease concentration. CFGM from overnight cultures of *S. aureus* (red), *E. faecalis* (purple), *S. pyogenes* (blue), *P. aeruginosa* (green), or *P. mirabilis* (black) of known concentration was diluted to  $1 \times 10^5$  to  $1 \times 10^8$  CFU/ml then assayed using HRP-CPI2 microbeads for 10 minutes. *S. pyogenes* was diluted 1:10 for this study because the beads detect it at a lower level. The amount of signal tag released was determined by the rate of color development using ABTS substrate. The signal demonstrated a concentration-dependent increase with bacterial concentration in the sample.

**Figure 3:** *In vitro* proteolytic activity of CFGM from bacterial wound isolates diluted to  $10^6$  CFU/ml (black) or  $10^4$  CFU/ml (grey). Diluted sample or a buffer control was mixed with HRP-CPI2-Trisacryl beads for 10 minutes and the amount

of HRP released was measured. The assay was performed in triplicate and bars represent the limits of the standard deviation from the mean.

**Figure 4:** Mass spectroscopy analysis of CPI2 fragments

(A) Mass spectroscopy data for limited proteolysis of the CPI2 peptide with *P. aeruginosa*, *E. faecalis*, *P. mirabilis*, *S. aureus*, HNE and undigested control peptide (MW 1501).

(B) Protease cleavage sites within the CPI2 peptide. Unlabeled CPI2 was exposed to human neutrophil elastase (HNE) or CFGM from *S. aureus* (SspA), *E. faecalis* (GelE), *S. pyogenes* (SpeB), *P. aeruginosa* (LasB), or *P. mirabilis* (ZapA) then analyzed by mass spectroscopy. The locations of predominant cleavage sites were determined by comparison of the mass of the proteolytic fragments to possible cleavage products.

**Figure 5:** PCR of protease genes found in *S. aureus* (V8), *P. aeruginosa* (LasB), *E. faecalis* (GelE), *S. pyogenes* (SpeB), and *P. mirabilis* (ZapA). The presence of the protease genes V8, LasB, GelE, SpeB, and ZapA was determined by polymerase chain reaction (PCR) by using primers specific to each gene to amplify a 290-320 base pair product for each gene. Genomic DNA was extracted from cell scraping off blood agar plates for each sample. The Masterpure™ Gram Positive (Epicenter Biotechnologies, Madison WI) or the Prepman™ (Applied Biosystem, Foster City, CA) DNA purification kits was used to isolate the DNA, 2ul of each genomic prep was used as a template for the PCR reactions. In all case the genes were found in 100% of strains examined suggesting that these are essential for virulence factors in each wound pathogen. All PCR reactions were performed for 25 cycles with a melting temperature of 92°C, anneal at 50°C and extension at 72°C in a MyIQ Single Color Real-Time PCR detection system (Bio-Rad, Hercules, CA).

**Figure 6:** A time course study was performed on Biotin-polyhistidine-CPI2S beads with a 5,10, 15 and 30 minute cleavage time. Purified V8 protease standard (EMD Biosciences) at 50 ng/ml was used to cleave the peptide. The beads were removed by centrifugation at the designated time and free dual tag

was measured in an ELISA assay using streptavidin -coated plates and NTA-HRP. Signal was measured by monitoring the increase in color (650 nm) after addition of TMB substrate. The time dependence of cleavage is shown in the graph versus a control of PBS buffer.

**Figure 7:** Improvement of substrate peptide to reduce cleavage by host proteases. Biotin-polyhistidine CPI2 peptide (circles) on beads shows a concentration dependent increase in signal from human neutrophil elastase (HNE) tested in the 0-1  $\mu\text{g/ml}$  range. The peptide on beads was exposed to HNE concentrations for 15 minutes and then the beads were filtered out of solution. The released tag was measured by ELISA and TMB color development. The graph shows the  $V_{\text{max}}$  at 650 nm for each concentration. The HNE cleavage site was removed from the CPI2 peptide in a newer version of the substrate: CPI2S. CPI2S shows reduced HNE activity (triangles) tested in the same manner in the same concentration range. Also shown is the reactivity of CPI2 and CPI2S peptide to purified V8 bacterial protease (diamonds and squares respectively) in the 0-1  $\mu\text{g/ml}$  range which is not greatly affected by the removal of the elastase cleavage site. MMP 2 and MMP 9 (0.5  $\mu\text{g/ml}$ ) were also tested on the CPI2 peptide. The results (not shown) yielded the same signal as a control indicating no CPI2 cleavage.

**Figure 8:** Human Neutrophil Elastase (HNE) was efficiently removed cation exchange (SP) resin. The samples that had the most elastase activity are shown before and after treatment with 500  $\mu\text{l}$  of a 50% slurry SP cation exchange resin. We determined that SP resin is very effective at removing HNE from a wound, suggesting that this method could be used to improve the signal to noise of the diagnostic.

**Figure 9:** Comparison of the mean proteolytic activity of infected and non-infected clinical samples. Error bars represent the 95% confidence interval of the mean.

**Figure 10: (a)** The protease activity was tested on 100 clinical samples from patients with non-healing wounds (>60days) attending the UMass Medical School Wound Clinic. We only considered patients with questionable infection status. This data shows a scatter plot of total CFU/ml vs. ng of peptide clipped normalized to 0.1 ml. Wounds that were presumed infected or truly uninfected by clinical signs were excluded from the study as well as any patient that had been treated with an enzymatic debriding agent for 30 days. The 45 ng of peptide cutoff was chosen to distinguish between infected ( $\geq 3 \times 10^5$  CFU/ml) and uninfected ( $< 3 \times 10^5$  CFU/ml) samples. **(b)** The clinical data represent: 69 true positives, 16 true negatives, 8 false negatives, and 7 false positives. The bacterial protease activity in samples from chronic wounds correlates with the concentration of bacteria (bioburden) found in the wound. The sensitivity of the test is 89.9% with a specificity of 69.6% and an overall accuracy of 85%.

**Figure 11:** Comparison of the mean proteolytic activity of “active” vs. “inactive” wound samples (based on the level of bacterial protease activity: above/below 45 ng of clip per 0.1 ml) and mean proteolytic activity of “infected” vs. “uninfected” wound samples (based on the bioburden: above/below  $3 \times 10^5$  CFU/ml). Activity is measured in nanograms of peptide clipped. Error bars represent the 95% confidence interval of the mean. **(a)** All 100 wound samples ; **(b)** Only 29 debrided wound samples.

FIGURES

Figure 1

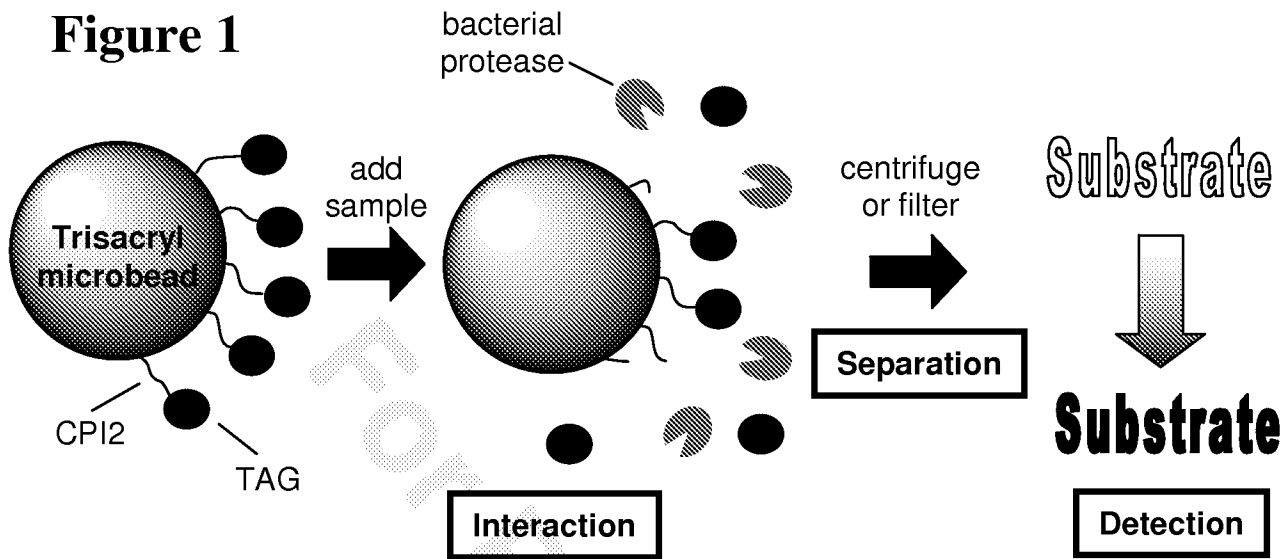


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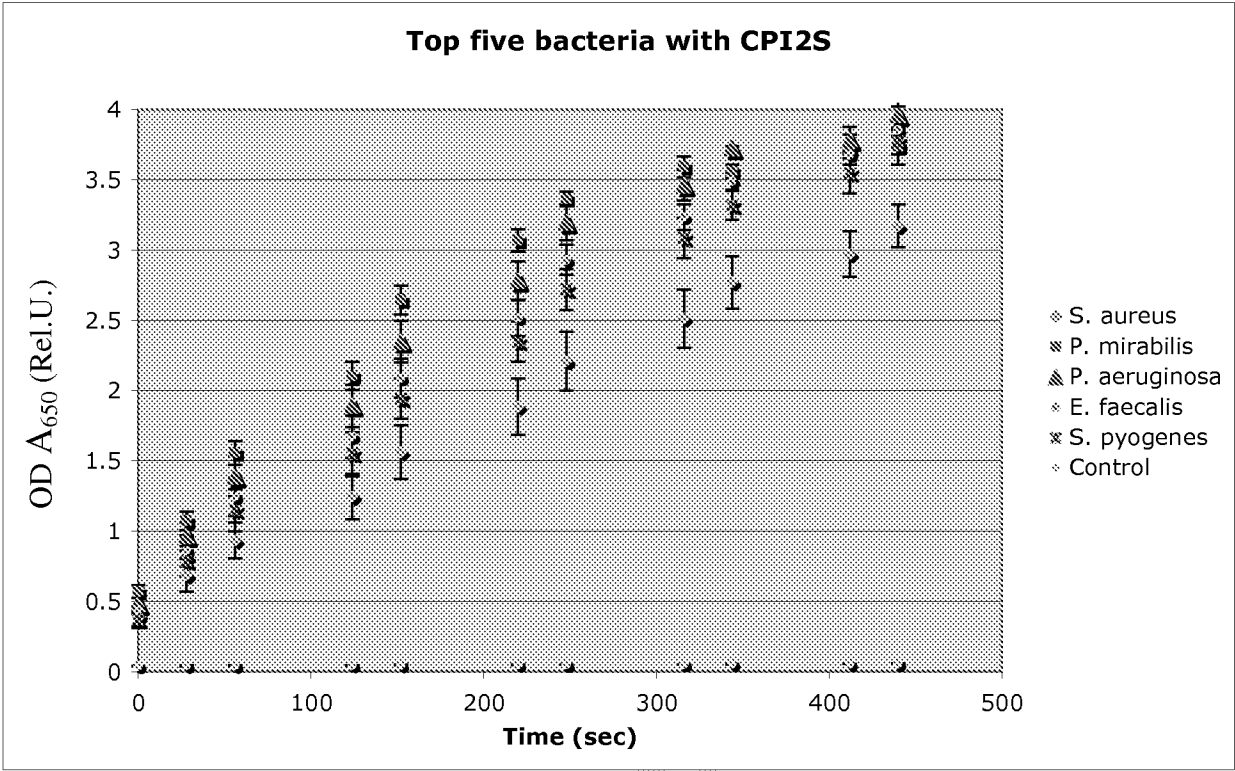


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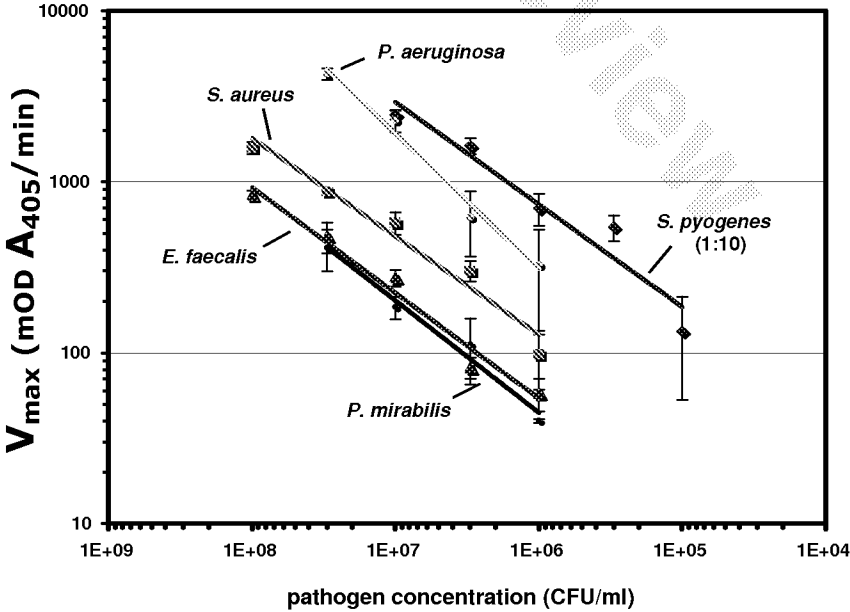




Figure 3

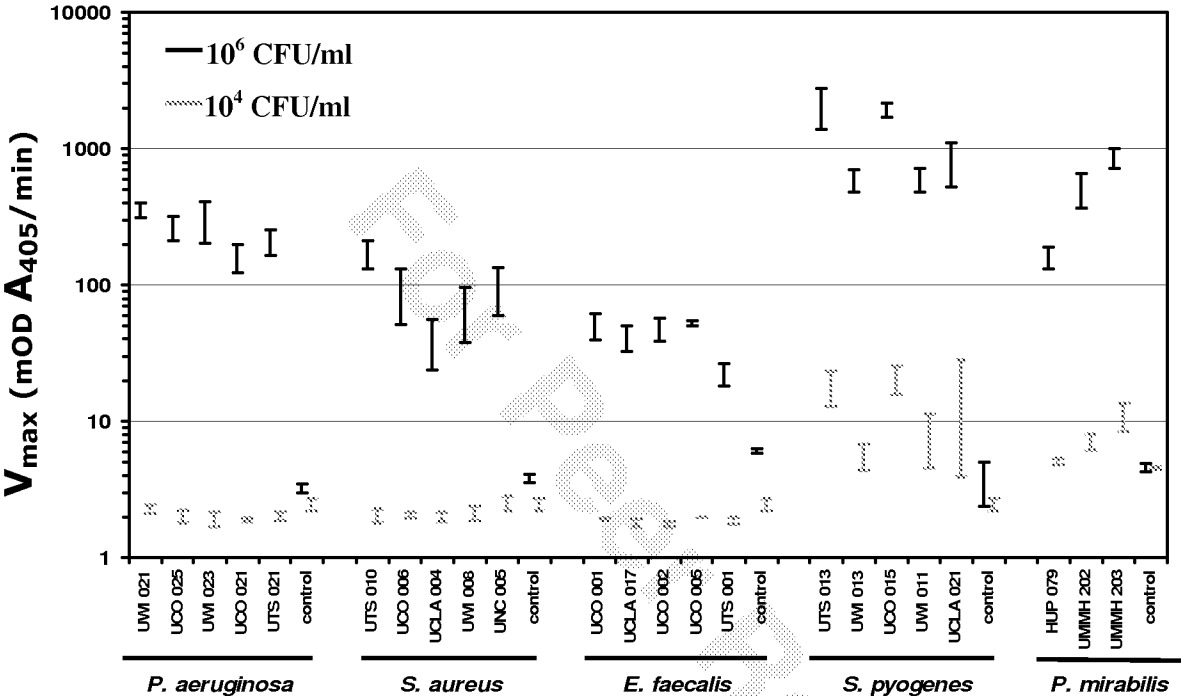
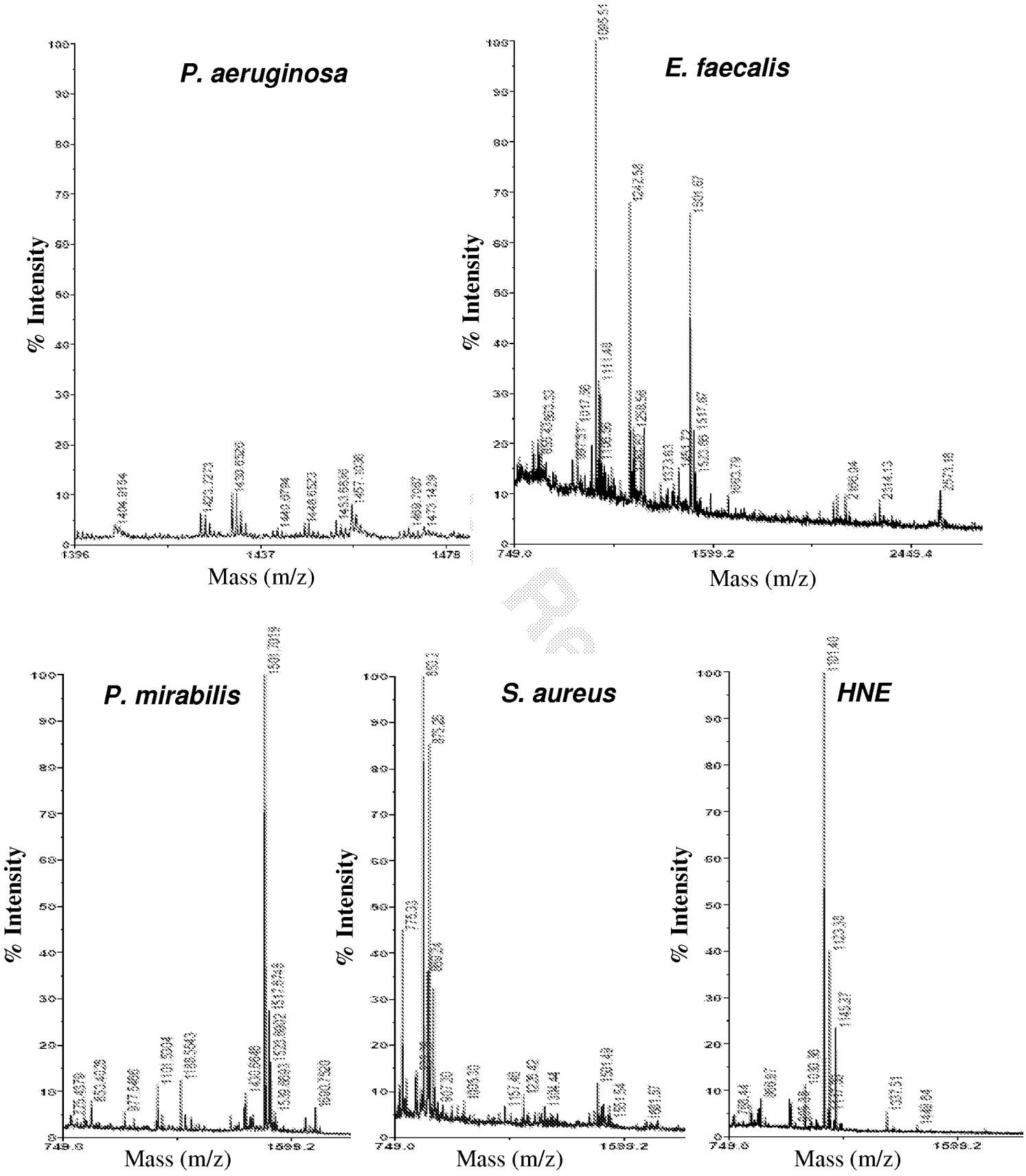


Figure 4 (a)



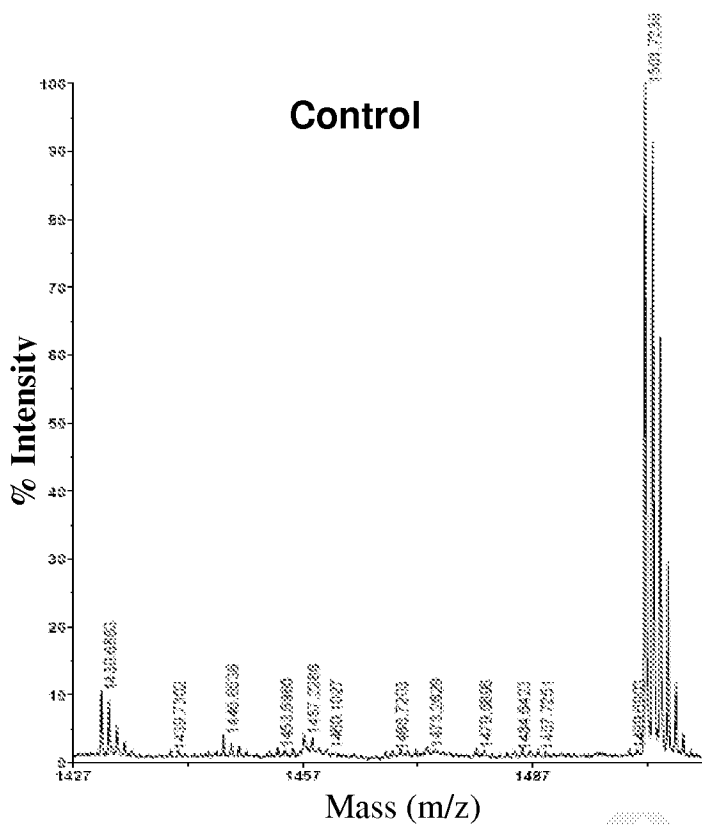


Figure 4 (b)

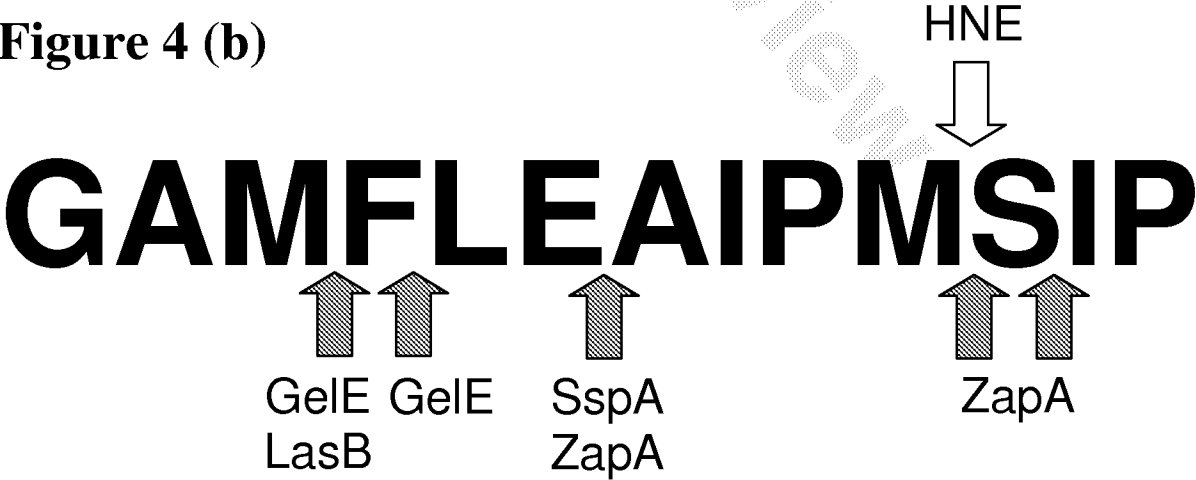


Figure 5

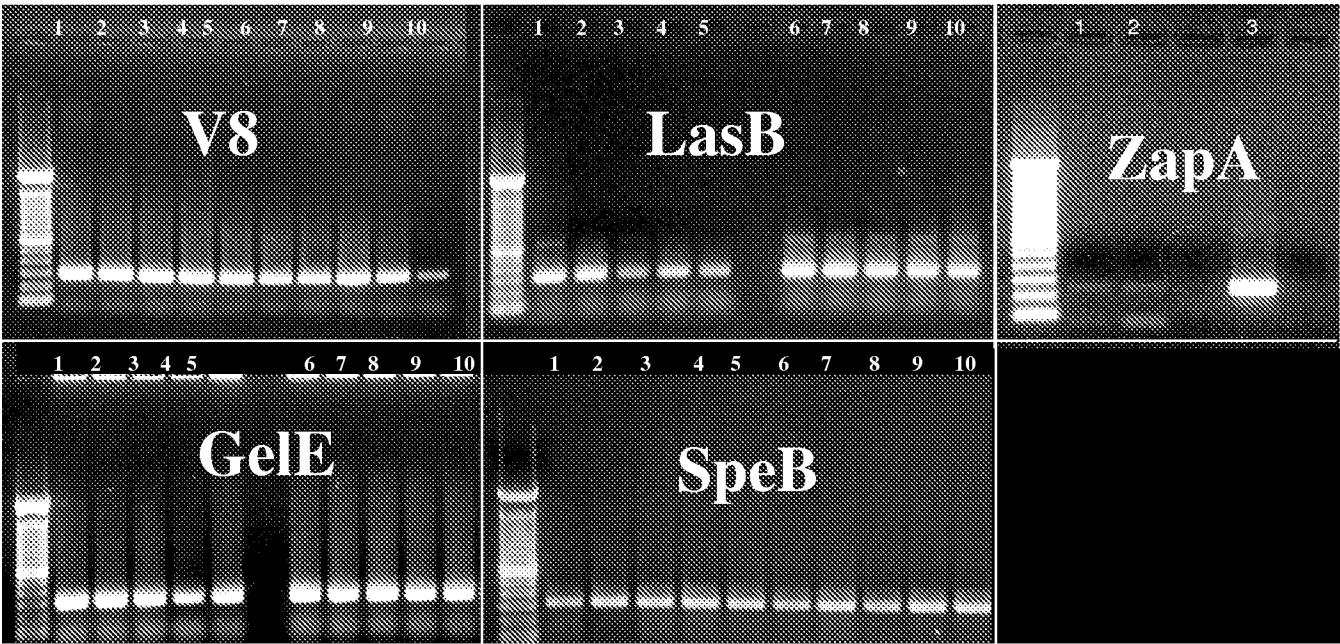


Figure 6

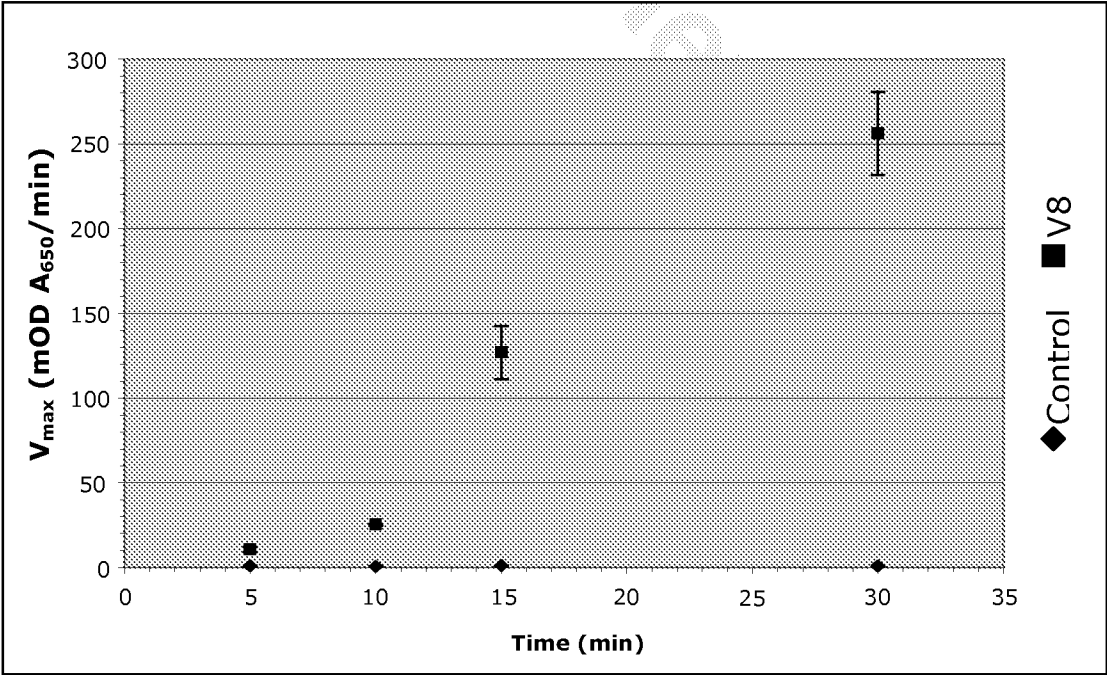


Figure 7

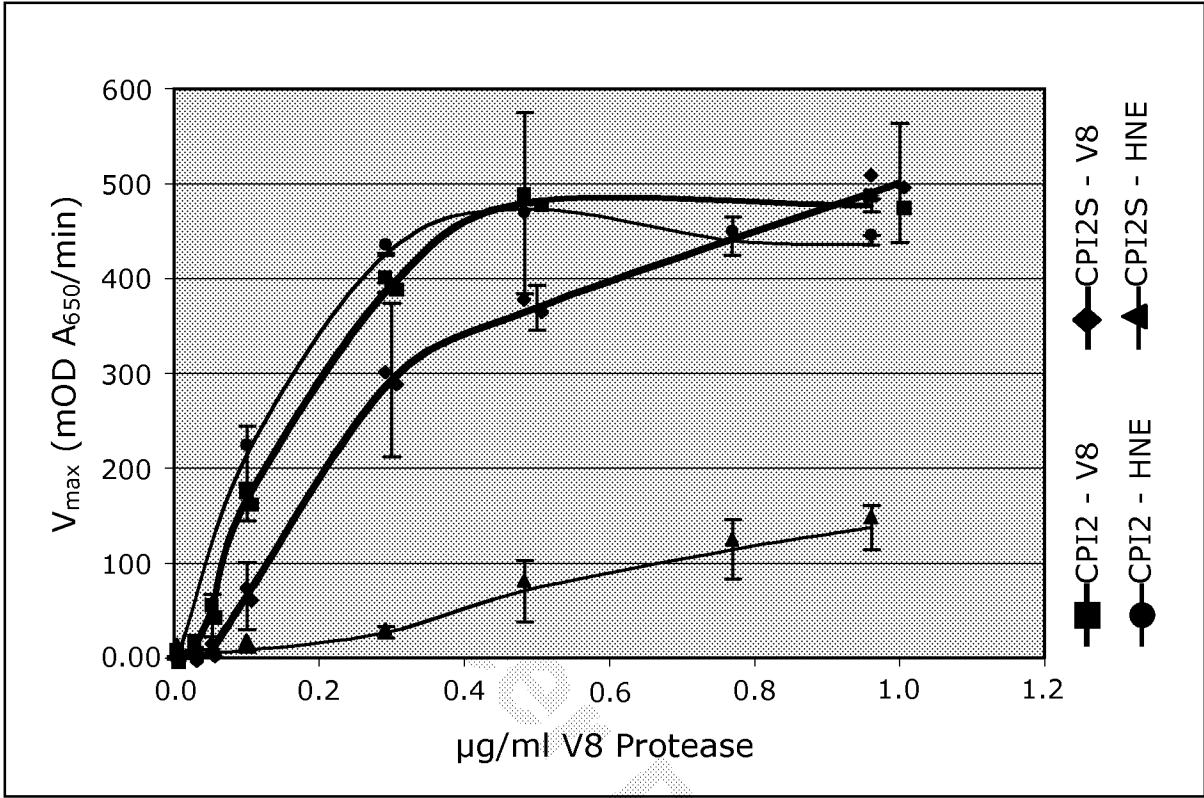


Figure 8

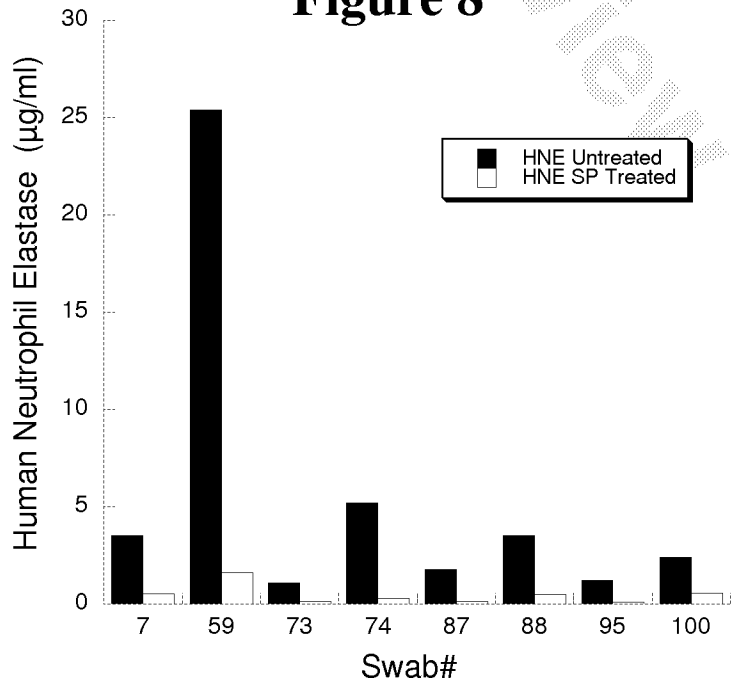


Figure 9

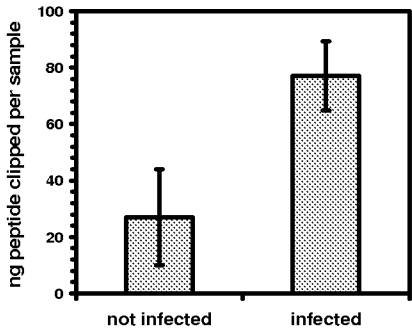


Figure 10 (a)

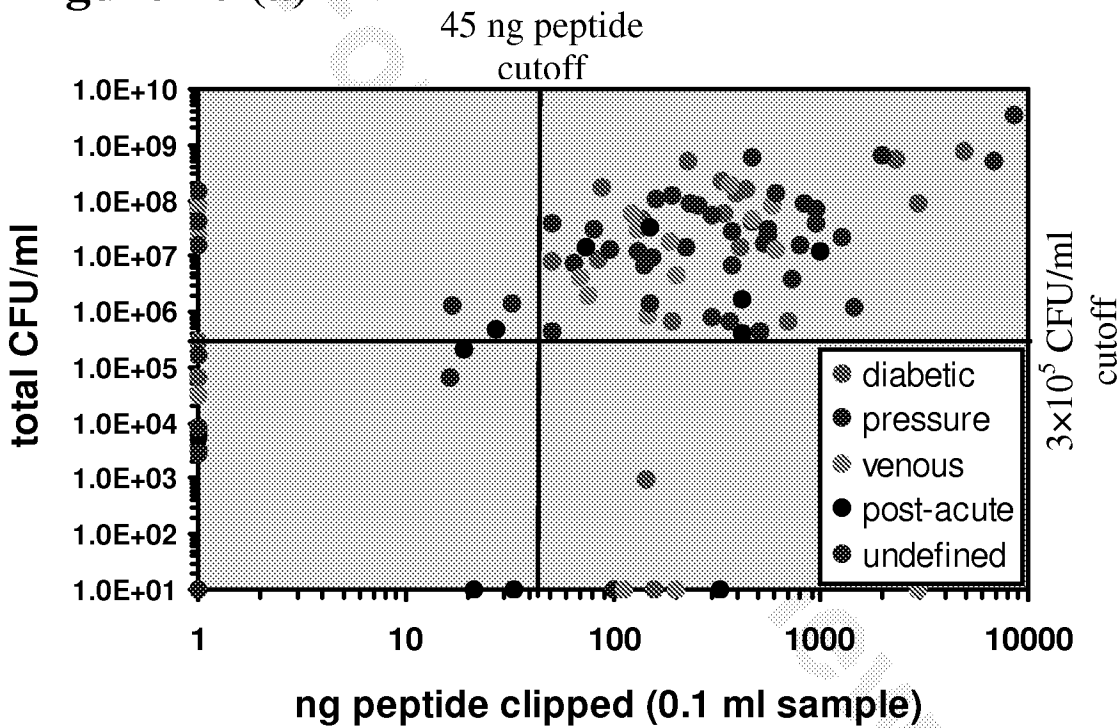


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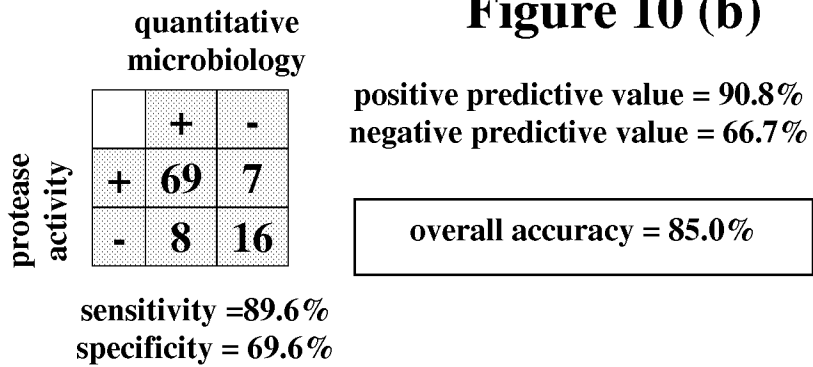


Figure 11 (a)

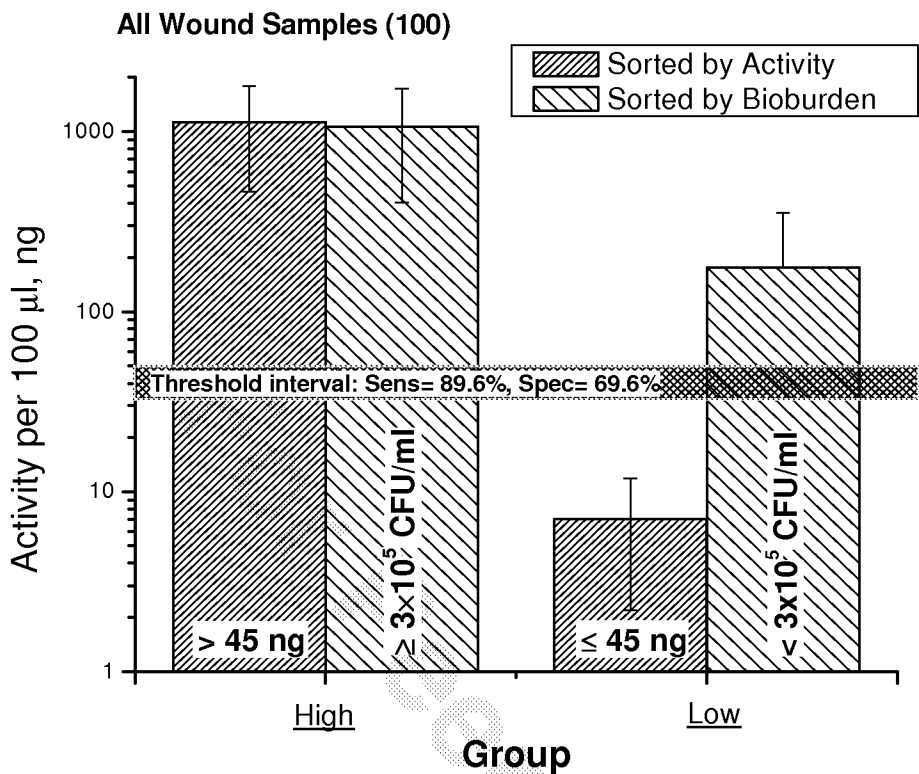


Figure 11 (b)

